Short Title: Shifts in temperature and *Batrachochytrium*

Shifts in temperature influence how *Batrachochytrium dendrobatidis* infects amphibian larvae

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Author contribution statement

ARB, JRR, and TRR originally formulated the idea. PWB designed the experiment and developed the methodology. PWB performed the experiment. PWB and MDB performed the molecular analysis. PWB and TRR performed the statistical analyses. ARB, JRR, TRR, and DHO obtained funding. PWB wrote the manuscript and other authors provided editorial advice.

Abstract:

Many climate change models predict increases in mean temperature, and increases in frequency and magnitude of temperature fluctuations. These potential shifts may impact ectotherms in several ways, including how they are affected by disease. Shifts in temperature may especially affect amphibians, a group with populations that have been challenged by several pathogens. Because amphibian hosts invest more in immunity at warmer than cooler temperatures and parasites may acclimate to temperature shifts faster than hosts (creating lags in optimal host immunity), researchers have hypothesized that a temperature shift from cold-to-warm might result in increased amphibian sensitivity to pathogens, whereas a shift from warm-to-cold might result in decreased sensitivity. Support for components of this climate-variability based hypothesis have been provided by prior studies of the fungus *Batrachochytrium dendrobatidis* (Bd) that causes the disease chytridiomycosis in amphibians. We experimentally tested whether temperature shifts before Bd exposure alter susceptibility to Bd in the larval stage of two amphibian species – western toads (*Anaxyrus boreas*) and northern red legged frogs (*Rana aurora*). Both host species harbored elevated Bd infection intensities under constant cold (15° C) temperature in comparison to constant warm (20° C) temperature. Additionally, both species experienced an increase in Bd infection abundance when shifted to 20° C from 15° C, compared to a constant 20° C but they experienced a decrease in Bd when shifted to 15° C from 20° C, compared to a constant 15° C. These results are in contrast to prior studies of adult amphibians that found increased susceptibility to Bd infection after a temperature shift in either direction, highlighting the potential for species and stage differences in the temperature-dependence of chytridiomycosis.

Keywords: amphibian declines, *Batrachochytrium dendrobatidis*, chytridiomycosis, climate variability hypothesis, infectious disease, temperature, *Rana aurora*, *Anaxyrus boreas*

Introduction

Climate change represents one of the greatest challenges to biodiversity and conservation because it might compromise ecosystem functions worldwide. Changes in climate have affected plant-animal interactions, predator-prey interactions and disease dynamics (Lafferty 2009, Rohr *et al.* 2011, Sheldon, Yang & Tewksbury 2011, Garcia *et al.* 2014). Changes to annual or seasonal mean temperatures often are used to predict climate-change-induced effects on disease risk (Paaijmans, Read & Thomas 2009, Paaijmans *et al.* 2010). However, many climate change models also predict increases in the frequency and magnitude of extreme weather events and increases in temperature variability at monthly to weekly timescales (Easterling *et al.* 2000, Meehl & Tebaldi 2004, Schar *et al.* 2004, Paaijmans *et al.* 2010, Rummukainen 2012). Yet few studies have investigated how increases in temperature variability affect disease dynamics despite the likelihood that such variability might differentially affect hosts and pathogens (Paaijmans *et al.* 2010, Ben-Horin, Lenihan & Lafferty 2012, Raffel *et al.* 2013, Bannerman & Roitberg 2014, Luis *et al.* 2014, Raffel *et al.* 2015). Ectotherms, such as amphibians, are particularly sensitive to climate change (Blaustein *et al.* 2010, Lawler *et al.* 2010, Shoo *et al.* 2011, Li, Cohen & Rohr 2013) and are experiencing disease-associated population declines and extinctions worldwide (Stuart *et al.* 2004, McCallum

2007, Rohr *et al.* 2008, Wake 2012), making them an ideal group to investigate the relationship between temperature shifts and disease risk.

Chytridiomycosis is an emerging infectious disease of amphibians caused by the aquatic chytrid fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Longcore, Pessier & Nichols 1999, Martel *et al.* 2013). Bd is widespread globally (Liu, Rohr & Li 2013, Olson *et al.* 2013) and is associated with worldwide amphibian population declines (Stuart *et al.* 2004, Skerratt *et al.* 2007). Moreover, models based on IPCC climate futures predict that Bd will shift to higher latitudes and altitudes due to increased environmental suitability in those regions under climate change, thus potentially affecting additional amphibian populations (Xie, Olson & Blaustein 2016).

The negative effects of Bd infection are more pronounced in post-metamorphic stages, often leading to death (Blaustein *et al.* 2005, Garner *et al.* 2009, Gervasi *et al.* 2013, Gervasi *et al.* 2017). In larvae, Bd infection can cause host mortality in some species (Blaustein *et al.* 2005, Garner *et al.* 2009). However the infection is localized to keratinized larval mouthparts, (Marantelli *et al.* 2004, McMahon & Rohr 2015) often resulting in sublethal effects including inhibited foraging capacity, reduced growth and development, altered predator avoidance, or changes to other behaviors (Han, Bradley & Blaustein 2008, Venesky, Parris & Storfer 2010, Buck *et al.* 2012, Gervasi *et al.* 2013). Additionally, larvae of many species are important members of aquatic communities and alterations to larval feeding have the potential to cascade through the aquatic ecosystem (Alford 1989, Brönmark, Rundle & Erlandsson 1991, Lamberti *et al.* 1992, Kupferberg 1997).

93 Temperature is considered one of the most important environmental factors
94 driving chytridiomycosis (Drew, Allen & Allen 2006, Bosch *et al.* 2007, Daskin, Alford
95 & Puschendorf 2011, Forrest & Schlaepfer 2011, Voyles *et al.* 2017). Bd is non-linearly
96 sensitive to temperature with an optimal growth range in culture between 17° and 25°
97 (Piotrowski, Annis & Longcore 2004, Rohr & Raffel 2010, Raffel *et al.* 2013) and a
98 temperature-dependent generation time of 4 to 10 days (Woodhams *et al.* 2008). The
99 upper thermal limit for Bd growth in culture is between 25°C and 28°C, with Bd
100 mortality occurring above 30°C (Longcore, Pessier & Nichols 1999, Piotrowski, Annis &
101 Longcore 2004). Bd has been shown to be reliably cleared from multiple amphibian
102 species by extended exposure to 30°C (McMahon *et al.* 2014). Its lower thermal limit is
103 below 4°C (Piotrowski, Annis & Longcore 2004). Additionally, life history strategies of
104 the pathogen can be altered by environmental temperature, where colder temperatures can
105 cause Bd zoosporangia to develop and mature more slowly (Voyles *et al.* 2012), but
106 produce more and longer-lived zoospores overall (Hyatt *et al.* 2007, Woodhams *et al.*
107 2008).

108 Because physiologies of both the host and pathogen are strongly influenced by
109 environmental temperature, climate change has been used to explain several major Bd
110 outbreaks and amphibian population declines, (reviewed in Li, Cohen & Rohr 2013, Rohr
111 *et al.* 2013). Yet, the host and pathogen are not expected to share a uniform response to a
112 given temperature (Brown *et al.* 2004, Paull, LaFonte & Johnson 2012, Rohr *et al.* 2013),
113 and thermal responses measured in constant-temperature artificial environments might
114 not reflect organism responses in more realistic variable-temperature environments.
115 Providing evidence of the lack of a uniform response between Bd and amphibians to

116 temperature shifts, Rohr and Raffel (2010) found a strong correlation between elevated
117 month-to-month temperature variability and Bd-associated amphibian population
118 declines of *Atelopus* spp. across Central and South America. Further support of the
119 relationship between chytridiomycosis and temperature variation has been provided by
120 laboratory studies. In one study, Cuban treefrogs (*Osteopilus septentrionalis*) displayed
121 reduced resistance to Bd infection when exposed to random daily temperature
122 fluctuations or when exposed to a temperature decrease after acclimation to a warmer
123 temperature (Raffel *et al.* 2013). Similar results were obtained in newts (*Notophthalmus*
124 *viridescens*) exposed to Bd, except both decreases and increases in temperature were
125 associated with elevated Bd abundance relative to abundances at constant temperatures
126 (Raffel *et al.* 2015).

127 The potential for temperature variability to increase disease severity in
128 amphibians was first postulated by Raffel *et al.* (2006) and has subsequently been
129 referred to as the "climate variability hypothesis" (Rohr & Raffel 2010). This hypothesis
130 posits that parasites acclimate to the new temperature more rapidly than their hosts,
131 leading to lags in host acclimation following a temperature shift that could make hosts
132 more susceptible to infection (Raffel *et al.* 2013). This hypothesis assumes that: 1)
133 pathogens acclimate to the new temperature faster than the host because of their
134 relatively smaller size and higher metabolic rate (Gillooly *et al.* 2001, Raffel *et al.* 2013);
135 and 2) both host and parasite acclimation responses lead to increased performance at the
136 new temperature, in accordance with the "beneficial acclimation hypothesis" of thermal
137 biology (Angilletta 2009). However, Raffel *et al.* (2006) also pointed out potential

138 complexities in acclimation of the ectotherm immune system that might lead to
139 alternative predictions.

140 According to the "*lag effect*" hypothesis (Raffel *et al.* 2006), changes in levels of
141 temperature-dependent immune parameters might simply lag behind environmental
142 temperature shifts (Fig. 1) because it takes time to produce necessary, or remove
143 unnecessary, immune cells from the host. For example, amphibians are expected to
144 require more immune cells at warmer temperatures to fight off faster-growing pathogens
145 (Maniero & Carey 1997), and lags in production of new immune cells could lead to sub-
146 optimal immunity following a temperature increase (Raffel *et al.* 2006). Conversely, the
147 amphibian immune system is expected to be down-regulated following a temperature
148 decrease (Macela & Romanovsky 1970), with the removal of mature white blood cells
149 determined by the rate of their respective half-lives (DeSantis & Strauss 1997, Janeway
150 2008). A lag in this process might lead to a brief period of elevated immune
151 responsiveness relative to an already cold-acclimated host. Thus, the "lag effect"
152 hypothesis predicts the opposite effect from the "climate variability hypothesis"
153 following a temperature decrease, at least on a short timescale. These mechanistic
154 hypotheses are not mutually exclusive, and it is unclear which effects might be more
155 important for a given host-parasite combination.

156 We tested the general prediction that an amphibian shifted to a new temperature
157 before Bd exposure would respond to infection differently than a host already acclimated
158 to the exposure temperature. We postulated that the direction of the effect would depend
159 upon the direction of the temperature shift, in accordance with the "lag effect" hypothesis
160 of Raffel *et al.* (2006). Given the differences in size between the host and the pathogen,

and associated physiological process rate differences, we assumed Bd would physiologically respond to the temperature shift faster than the host, such that an idealized host-immune response to Bd exposure would temporarily lag behind the temperature shift. Thus, we predicted that a temperature shift from cold-to-warm would result in an *increase* in susceptibility to Bd exposure, whereas a temperature shift from warm-to-cold would result in a *decrease* in susceptibility to Bd exposure. To test these predictions, we quantified susceptibility to Bd by measuring infection abundance after exposure to the pathogen.

Materials and Methods

To examine the how temperature shifts may alter larval amphibian infection dynamics, we selected two species of amphibian hosts, the northern red legged frog (*Rana aurora*) and the western toad (*Anaxyrus boreas*). Both species have been observed in the field with Bd infections (Pearl *et al.* 2007, Muths, Pilliod & Livo 2008, Piovia-Scott *et al.* 2011) and both species are susceptible to chytridiomycosis (Han, Bradley & Blaustein 2008, Gervasi *et al.* 2013). To ensure that the animals used in our experiment were not previously infected with Bd, amphibians were collected as eggs from natural oviposition sites. Red legged frog eggs were collected from a permanent pond located near Florence, Oregon, USA (Lincoln County, elevation 12 m; latitude/longitude: 44.088/-124.123) in the Oregon Coast Range on 11-Feb-2012. Western toad eggs were collected from Little Three Creeks Lake (Deschutes County, elevation 2,000 m; latitude/longitude: 44.009/-121.643) in the Cascade Range on 9-Jul-2011. Immediately after collection, eggs were transported to a laboratory at Oregon State University where

they were maintained at 14° C, under a 12-12 photoperiod in 40-liter aquaria filled with dechlorinated water. Upon hatching, larvae were maintained at a density of approximately 200 individuals per aquarium and fed *ad libitum* a mixture of Tetramin fish food and ground alfalfa pellets (1:3 ratio by volume). Water was changed every seven days. The 40-day trials for each species were not run concurrently, but identical protocols were used for both species and both trials consisted of individuals of identical larval stage (Gosner stage 26).

Acclimation Period

Independent trials for each host species began with a 20-day acclimation period with 80 (Gosner stage 26) larvae randomly selected, and individually placed into 80 plastic 500-mL containers where they were housed for the duration of the acclimation period and experiment. Each container was filled with 14° C dechlorinated water and covered with a lid to help maintain water temperature and limit evaporation. Each container had 2-mm diameter holes drilled between the water line and the lid to allow air circulation into the container. Pairs of containers were then placed within 40 individual temperature-controlled chambers (to ensure independent replication of the temperature treatments) that were set at 15° C to avoid cold-shocking the larvae. Each temperature-controlled chamber was independently controlled via its own thermostat and the interior measured approximately 37 cm deep x 21 cm wide x 13 cm in height. Half of the 40 temperature-controlled chambers were then randomly selected to begin the acclimation period at 20° C (warm treatment) and the other half were kept at 15° C (cold treatment). The placement of temperature chambers within the laboratory was randomized, as was the placement of 500-mL containers within each temperature chamber.

Temperature Shifts

On day 20 of the experiment, half of the temperature chambers in each of the two acclimation temperatures (15° C and 20° C) were randomly selected to undergo a temperature shift, either from 20° to 15° C or from 15° C to 20° C. The other half of the temperature chambers underwent no shift in temperature. Thus, each of the temperature chambers was subjected to one of four temperature treatments: a constant 15° C (cold) throughout the experiment; a constant 20° C (warm) throughout the experiment; a temperature shift from 15° C to 20° C (cold-to-warm); or a temperature shift from 20° C to 15°C (warm-to-cold).

Bd exposure

On day 24, one of the two 500-mL containers within each temperature-controlled chamber was randomly selected to undergo a Bd-exposure treatment and the other was selected as a control. Larvae in the Bd-exposure treatment were exposed to a single inoculate of Bd strain JEL 274, which was grown in pure culture on 1% tryptone agar in 10-cm diameter Petri dishes. This Bd strain was selected as it is one of the more virulent strains associated with major amphibian populations declines (Rosenblum *et al.* 2013). The Petri dishes were inoculated with liquid culture 10 days before the start of the experiment and incubated at 15° C. To harvest the zoospores, 10 plates were flushed with 15 mL of 15° C dechlorinated water and remained undisturbed for 10 minutes. The plates were scraped with a rubber spatula to release the zoospores and sporangia adhering to the agar. The inoculum from each plate was then pooled in a beaker and the number of moving zoospores was determined using a hemocytometer. After quantifying the zoospore concentration, the inoculum was diluted to 10,000 zoospores/mL. Individuals in

the Bd-exposed treatments were exposed to 10 mL of inoculum transferred into the 500-mL container housing an individual larva. Control individuals were exposed to 10 mL of sham inoculum lacking the Bd culture (made from 1% tryptone sterile agar plates following the same methods), similarly transferred into the 500-mL container housing each larva. Thus, the individual larva underwent their exposure treatment on day 24, four days after the water temperature shift for chambers in the two temperature shift treatments.

During the 40-d trial, survival and metamorphic status were checked daily. Water for each 500-mL container within the temperature chambers was changed every 12 days and consisted of dechlorinated water of the same temperature (15° C and 20° C). Individuals that survived until the end of the trial (i.e., day 40) were euthanized in a 2% solution of MS-222, and then preserved in 95% ethanol. Individuals that reached metamorphosis (Gosner stage 42: emergence of forelimbs) were euthanized, measured, and preserved as previously described.

Determining infection status

We used quantitative polymerase chain reaction (qPCR) to determine infection status and quantify Bd-infection intensity of all individuals in the Bd-exposure treatments. Additionally, we investigated Bd-infection status in eight randomly selected control individuals per species. To sample the individuals for Bd, we extracted whole mouthparts of the larvae using sterile dissection scissors. We conducted qPCR using an ABI PRISM 7500 sequencer (Applied Biosystems) according to the methods of Boyle *et al.* (2004) except that we used 60 µL of Prepman Ultra (Applied Biosystems, Carlsbad, California, USA), instead of the 40 µL in the DNA extraction. All samples were run in

253 triplicate and averaged.

254 *Statistical Analyses*

255 Each temperature-controlled chamber was an experimental unit (whole plot) and
256 the pairs of containers within each chamber acted as subplots. The whole plots were
257 subjected to one of four temperature regimes consisting of a Bd-exposure temperature
258 combined with a temperature shift status (constant cold, constant warm, shifted to cold,
259 and shifted to warm). Further, subplots were subjected to one of two exposure treatments
260 (Bd exposed and Bd unexposed).

261 Survival was compared between temperature treatments for western toad
262 larvae with a Cox proportional hazards model (Cox 1972) using TIBCO Spotfire S+
263 version 8.1. The model consisted of the main effects of the temperature treatment,
264 temperature shift status (constant versus shifted), and an interaction between the
265 two variables. Due to losses of western toad larvae prior to the application of the
266 exposure treatment, we lacked the power to statistically compare survival in
267 western toad larvae between the Bd exposure treatments

268 Bd infection abundance (Bd genomic equivalents) among temperature treatments
269 and between host species was analyzed using R version 3.11. We used a zero-inflated
270 negative-binomial generalized linear model (function 'zeroinf' in package 'pscl) as
271 described by Raffel *et al.* (2010), which includes a zero-inflation component that models
272 infection status as a binomial process (binomial distribution with a logit link) and a count
273 component that models infection intensity as a negative binomial process (negative
274 binomial distribution with a log link). Our full model investigated the effects of all of the
275 explanatory variables including host species, exposure temperature, temperature shift

276 status, and all two- and three-way interactions on Bd (*Batrachochytrium dendrobatidis*)
277 abundance. Interpretation of this analysis required further reduced models to investigate
278 the effect of exposure temperature and temperature shift for each species (species model)
279 and the effect of temperature shift for each Bd-exposure temperature and host species
280 combination (Bd-exposure temperature model).
281
282 **Results**
283 Survival differences were not detected between exposure temperatures (Cox,
284 Z = -1.099, $p = 0.27$) or temperature shift status (Cox, Z = -0.277, $p = 0.78$) in Bd-
285 exposed western toad larvae. We were unable to detect survival differences in red
286 legged frog larvae, as only one individual larva experienced mortality after
287 application of the exposure treatment (Table S1).
288
289 *Infection Abundance*
290 We detected a host species by temperature shift interaction ($\chi^2_1 = 3.83$, $p = 0.050$;
291 Table S2) and a Bd-exposure temperature by temperature shift interaction ($\chi^2_1 = 7.50$, $p =$
292 0.006; Table S2). We investigated these interactions with reduced models to investigate
293 effects on Bd abundance at the levels of species and exposure temperature.
294 Red legged frog larvae had higher Bd abundance when they were exposed to
295 infection at 15° C when compared to 20° C ($\chi^2_1 = 3.88$, $p = 0.049$; Fig. 2). The main
296 effect of temperature shift was marginally significant in the reduced species model
297 analysis ($\chi^2_1 = 3.50$, $p = 0.061$), but there was a significant effect of temperature shift for
298 individuals exposed at 20° C in the reduced Bd-exposure model ($\chi^2_1 = 5.7$, $p = 0.017$),

299 with individuals shifted from 15° C to 20° C having higher Bd abundance than red legged
300 frog larvae experiencing constant 20° C (Fig. 2). In contrast, there was no evidence that a
301 temperature shift influenced Bd infection when red legged frog larvae were exposed to
302 Bd at 15° C ($\chi^2_1 = 0.6$, $p = 0.4$; Fig. 2). There was no statistically significant interaction
303 between exposure temperature and temperature shift for red legged frog larvae ($\chi^2_1 = 2.4$,
304 $p = 0.13$).
305 We detected an interactive effect of exposure temperature and temperature shift
306 on Bd abundance in western toad larvae ($\chi^2_1 = 5.2$, $p = 0.023$). This was driven by
307 elevated Bd abundance in individuals under the constant 15° C temperature when
308 compared to individuals that experienced a temperature shift from 20° to 15° C, but no
309 evidence of an effect of shifting temperature from 15° C to 20° C (Fig. 2). There were no
310 main effects of exposure temperature ($\chi^2_1 = 0.50$, $p = 0.5$) or temperature shift ($\chi^2_1 < 0.01$,
311 $p = 0.9$) on Bd abundance in western toad larvae. Further, when investigating the
312 exposure temperatures individually in the reduced Bd-exposure model, there was no
313 evidence that a temperature shift influenced Bd infection in western toad larvae after
314 exposure to Bd at 15° C ($\chi^2_1 = 3.4$, $p = 0.066$) or 20° C ($\chi^2_1 = 2.5$, $p = 0.11$).
315 We failed to find evidence that the two host species differed in response to
316 exposure to the pathogen, leading us to conclude that general patterns for both species
317 were similar (Fig. 2). Both species experienced an increase in Bd abundance when
318 shifted to 20° C compared to a constant 20° C, and both generally experienced a decrease
319 in Bd abundance when shifted to 15° C compared to a constant 15° C. Additionally, both
320 host species experienced elevated Bd abundance in the constant 15º C treatment when
321 compared to the constant 20° treatment.

All red legged frog individuals survived until the end of the experiment but a number of western toad individuals died or metamorphosed earlier (Table S2). We therefore assessed the possibility that the timing of Bd sampling or the proximity of a larva to metamorphosis might drive observed patterns of Bd abundance in western toads. The model for Bd abundance on western toads was not significantly improved by adding either a variable coding whether individuals were near metamorphosis when sampled (χ^2_1 = 4.00, p = 0.150) or a covariate indicating the sampling date (χ^2_1 = 3.33, p = 0.068). Furthermore, neither variable qualitatively changed the contribution of exposure temperature or temperature shift status to the model. Therefore, we omitted both covariates from the final model for western toads.

Discussion

Our results suggest that Bd infection dynamics in larval amphibians can be affected by a shift in water temperature before host exposure to the pathogen, and that the direction of temperature shift determines the outcome of Bd exposure. Similar patterns were observed for the two host species when comparing individuals exposed to constant versus shifted temperatures. A shift from the warm temperature to the colder temperature was associated with a significant decrease in Bd abundance in western toad larvae and no significant decrease in red legged frog larvae. Likewise, a shift from the cold temperature to the warmer temperature significantly increased Bd abundance in red legged frog larvae and had no significant effect in western toad larvae. Importantly, we detected the effects of temperature shifts despite the host having a four-day head start on acclimating to the Bd exposure temperature relative to the pathogen. This suggests that we are likely

345 underestimating the strength of these effects and that their magnitudes might have been
346 larger if the host and pathogen experienced the shifts concurrently.

347 Amphibian species do not all respond similarly to a given Bd exposure. Species-
348 level differences in host tolerance to Bd infections have been well documented under
349 controlled laboratory conditions (Searle *et al.* 2011, Gervasi *et al.* 2017). Under natural
350 conditions, pathogen tolerance within a species may be affected by biotic factors such as
351 inter- and intra-specific interactions, proximity to metamorphosis, or life stage (Parris &
352 Cornelius 2004, Rachowicz & Vredenburg 2004, Blaustein *et al.* 2005, McMahon &
353 Rohr 2015) or abiotic factors such as temperature, season, or resource availability (Berger
354 *et al.* 2004, Raffel *et al.* 2010). For some susceptible host species, temperature-shift
355 induced changes in Bd abundance might alter the outcome of infection by either pushing
356 *Bd* abundance over or under a tolerance threshold. Such changes in relation to pathogen
357 abundance and pathogen tolerance may result in altering the strength of negative effects
358 of Bd infection. For example, temperature shifts in synergy with Bd infection may result
359 in either positive or negative effects on growth and development rates, foraging
360 efficiency, or predator avoidance (Parris & Cornelius 2004, Parris, Reese & Storfer 2006,
361 Venesky, Parris & Storfer 2010, Venesky, Wassersug & Parris 2010).

362 We hypothesized that hosts exposed to a shifted temperature would respond to
363 infection differently than hosts exposed to a constant temperature, and under the
364 framework of the "lag effect" hypothesis (Raffel *et al.* 2006, Rohr & Raffel 2010), the
365 direction of the temperature shift would differentially affect infection severity. We
366 predicted that a temperature shift from cold-to-warm would leave hosts in a temporarily
367 immune-compromised state and result in an elevated Bd abundance after exposure when

368 compared to hosts exposed to a constant warm temperature. Conversely, we predicted
369 that a temperature shift from warm-to-cold would provide hosts with a temporarily
370 elevated-immune responsiveness and result in a decrease in Bd abundance after exposure
371 when compared to hosts exposed to a constant cold temperature. Our results were
372 consistent with predictions of the "lag effect" hypothesis, and were generally consistent
373 with previous studies showing that a shift in temperature influences Bd infection in
374 postmetamorphic amphibians (Raffel *et al.* 2013, Raffel *et al.* 2015). In particular, our
375 finding of decreased resistance to infection following a temperature increase (relative to
376 warm-acclimated individuals) mirrored a laboratory study of post-metamorphic red-
377 spotted newts (*Notophthalmus viridescens*), where juvenile newts exhibited decreased Bd
378 resistance following a shift from 15° C to 25° C (Raffel *et al.* 2015). These findings of
379 fluctuating temperature effects on Bd infection across four anuran taxonomic groups and
380 life-stages suggest that effects of temperature shifts and Bd-related chytridiomycosis
381 susceptibility might be widespread within amphibians. However, our finding of increased
382 resistance to Bd infection following a temperature decrease (relative to cold-acclimated
383 individuals) was opposite the pattern observed in red-spotted newts and Cuban treefrogs
384 (Raffel *et al.* 2013, Raffel *et al.* 2015) These contrasting results suggests that there are
385 important among-taxa or among-stage differences in the underlying mechanisms driving
386 the effects of temperature fluctuation on Bd infection; whereas our results in pre-
387 metamorphic life-stage of western toads and red legged frogs are consistent with the "lag
388 effect" hypothesis, results of similar studies investigating post-metamorphic red-spotted
389 newts and Cuban treefrogs support the "climate variability hypothesis."

We observed differences in Bd abundance on our two amphibian species at the two constant temperature treatments. Higher Bd abundances were observed for both host species under the constant cold temperature treatment compared to the constant warm temperature treatment. These results are consistent with previous experiments that showed increased Bd abundance (Raffel *et al.* 2015) and Bd-induced mortality (Kilpatrick, Briggs & Daszak 2010, Murphy, St-Hilaire & Corn 2011, Raffel *et al.* 2015) associated with lower temperatures. This is despite Bd growing best in culture at about 23° C, which is much closer to the warm than cold temperature treatments in this experiment (Piotrowski, Annis & Longcore 2004, Woodhams *et al.* 2008). This might be because the larval immune response to Bd infection increases with increasing temperatures at a faster rate than the infectivity or growth rate of Bd (Raffel *et al.* 2013), or alternatively because of the differences between the growth rate of Bd in culture compared to the growth rate on host tissue (Venesky *et al.* 2013). Our results provide further evidence to suggest patterns of Bd growth in culture differ from patterns of Bd growth on a host and that it is important to assess the host-parasite interaction when predicting effects of climate and climate change on disease risk.

Alternatively, differences in Bd abundance between the two constant temperature treatments might be due to temperature effects on the pathogen rather than the host (Woodhams *et al.* 2008, Voyles *et al.* 2012). The Bd was cultured at 15° C; it is possible that the temperature shift experienced by the pathogen in the warm exposure treatment caused the depressed Bd abundances observed in both host species compared to the elevated Bd abundance in the cold exposure temperature treatment. A decrease in temperature may cause an increase in the number of Bd zoospores released from

zoosporangia (Hyatt *et al.* 2007, Woodhams *et al.* 2008), however the effect of a similar increase in temperature on Bd physiology is unclear.

In conclusion, our results provide additional evidence for climate variability affecting Bd infection in amphibians but suggest important among-taxa differences in the directionality of these effects. Our finding of increased host resistance to infection following a temperature decrease is consistent with the "lag effect" hypothesis of Raffel *et al.* (2006) but contradicts components of the "climate variability hypothesis", which has been proposed as an explanation for patterns of Bd-associated amphibian population declines (Rohr & Raffel 2010, Raffel *et al.* 2013, Raffel *et al.* 2015). Our study highlights the complexity that temperature plays in determining the outcome of Bd-amphibian interactions and the role that a fluctuating temperature might play in altering these interactions. Furthermore, this study increases the diversity of amphibian species and stages that have been shown to exhibit thermal acclimation effects on disease, and the broad generality of this pattern across four disparate taxa suggests that fluctuating-temperature effects on amphibian infection may be widespread. Accurately predicting the effects of global climate change on infectious diseases, such as chytridiomycosis will require further understanding of how infectious agents respond to heterogeneity in temperatures and temperature fluctuations.

Acknowledgments

All applicable institutional and national guidelines for the care and use of animals were followed; this research was conducted under Oregon State University IACUC animal care and use permit 3917. Collection of amphibian eggs was approved by the

Oregon Department of Fish and Wildlife (Oregon Scientific Taking Permit #006-12 issued to ARB). We thank S. Bauer, E. Davis, E. Hunt, A. Koosman, B. Meyers, M. Ouspenskaya, E. Peseke, V. Raffeale, and C. Rains for their help performing the experiment, K. Boersma for her help with the experimental design, and E. Boersley for her support and assistance. Additionally we thank J. Spatafora, V. Weis, and the Center for Genome Research and Biocomputing at Oregon State University for providing laboratory space for qPCR. This research was supported by grants from the National Science Foundation (EF-1241889), National Institutes of Health (R01GM109499, R01TW010286), U.S. Department of Agriculture (NRI 2006-01370, 2009-35102-0543), and U.S. Environmental Protection Agency (CAREER 83518801) to JRR and NSF grant IOS 1121529 to TRR. Support was provided by the U.S. Forest Service Pacific Northwest Research Station, Corvallis, Oregon to DHO.

Conflict of Interest: The authors declare that they have no conflict of interest.

Supporting Information

Additional supporting information may be found in electronic supplementary material for this article.

Literature Cited

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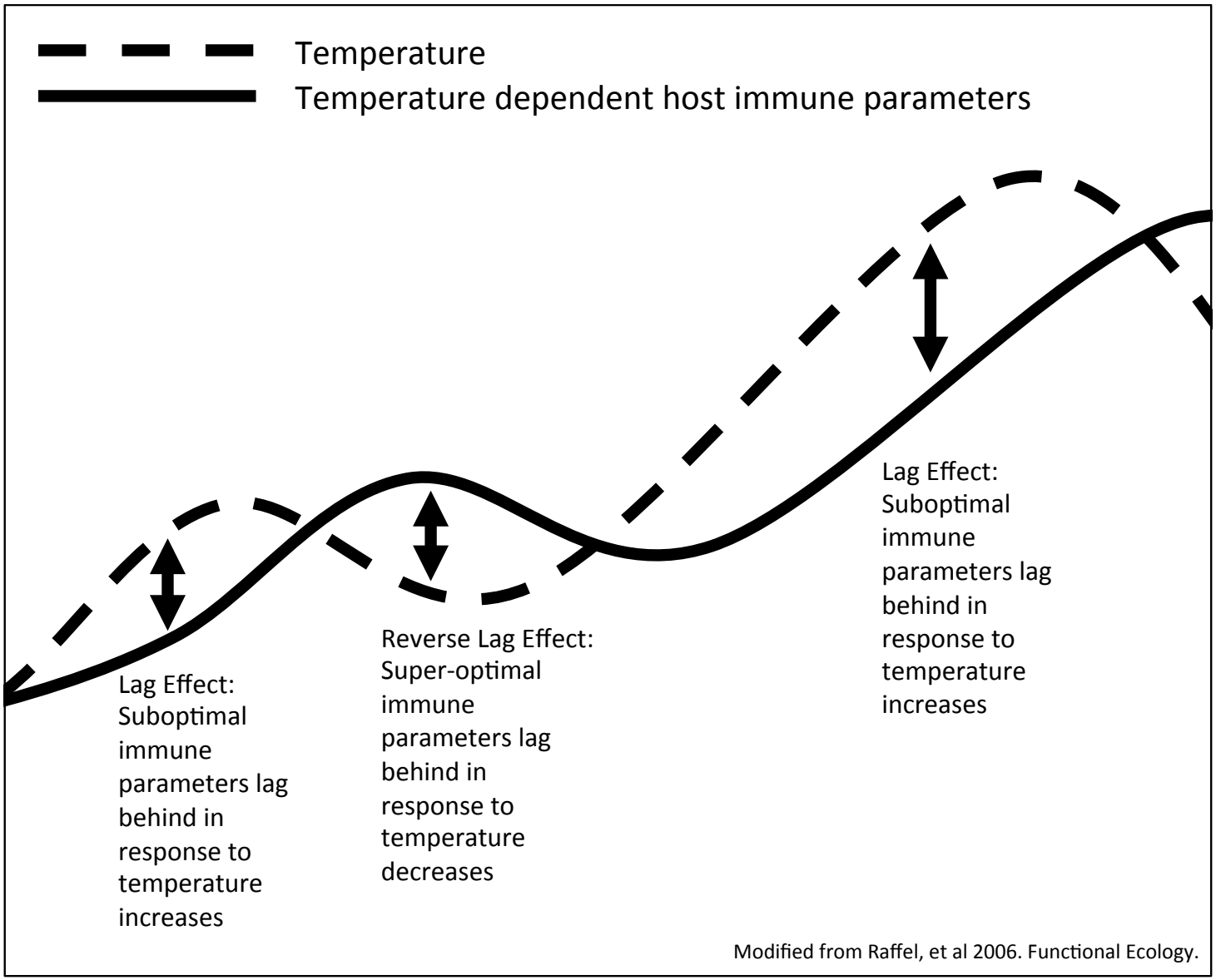
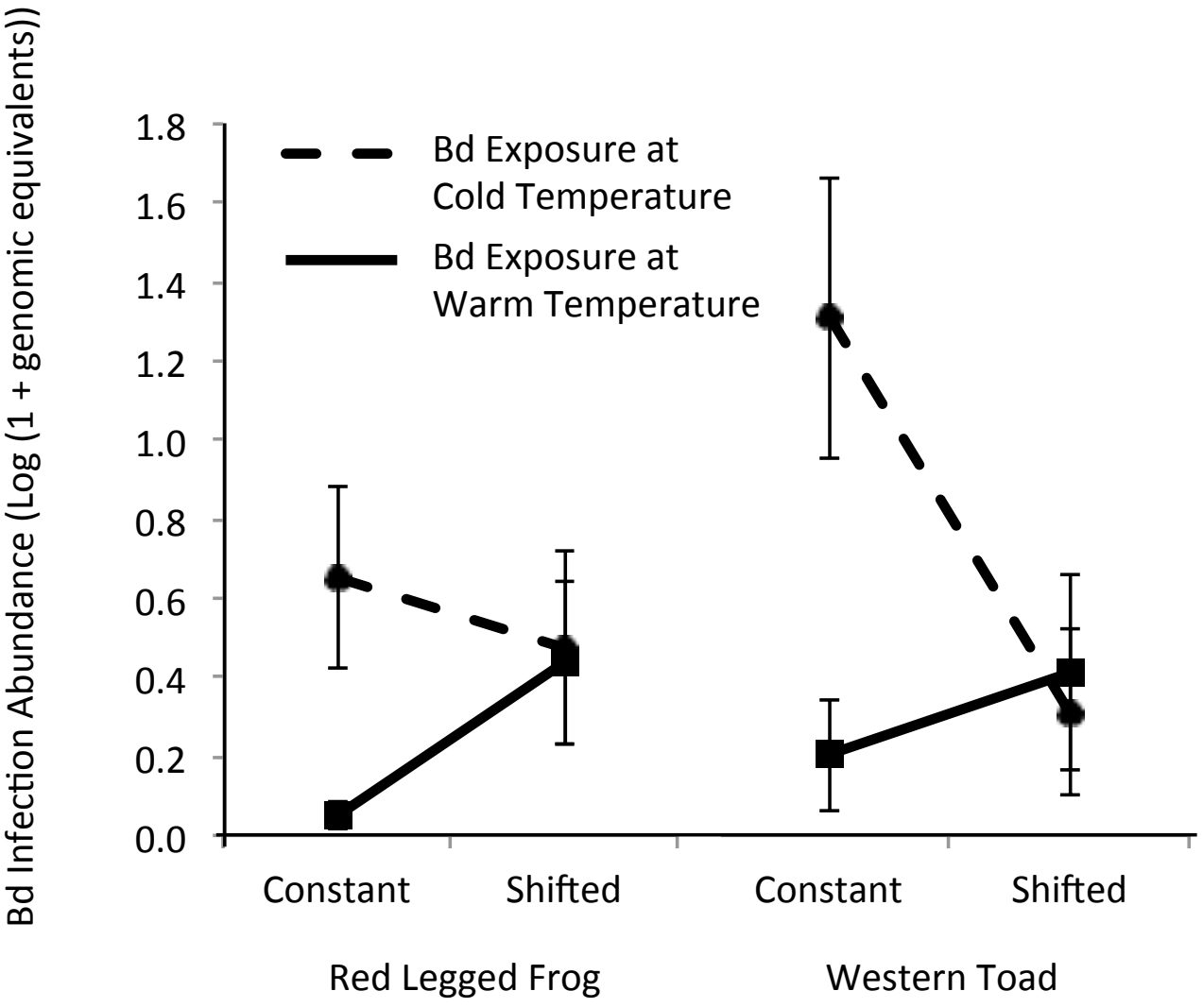
695

696

697 **Fig 1.** Hypothesized lag effect showing the relationship between fluctuating
698 temperatures (over days to weeks) and the optimal levels of a hypothetical
699 temperature-dependent host immune parameter. The immune parameter follows and
700 lags behind temperature changes – resulting in periods of a compromised immune
701 status after a temperature increase, and resulting in an over-active (or unnecessarily
702 costly) immune status after a temperature decrease. Modified from Raffel *et al*.
703 (2006).
704
705 **Fig 2.** Mean *Batrachochytrium dendrobatidis* (Bd) infection abundance (± SE)
706 measured at death, or at euthanasia 16-days after Bd exposure, in both western toad
707 (*Anaxyrus boreas*) larvae and red legged frog (*Rana aurora*) larvae from Oregon,
708 USA, and between the two temperatures at the time of Bd-exposure (cold [15° C]
709 versus warm [20° C]) and between larvae having experienced either a constant or
710 shifted temperature. Bd infection abundance is quantified as the log (1 + Bd genomic
711 equivalents) per excised larval mouthparts of all individuals exposed to the pathogen.
712


Temperature
Temperature dependent host immune parameters
Lag Effect: Suboptimal immune parameters lag behind in response to temperature increases
Reverse Lag Effect: Super-optimal immune parameters lag behind in response to temperature decreases
Lag Effect: Suboptimal immune parameters lag behind in response to temperature increases
Modified from Raffel, et al 2006. Functional Ecology.
Time in Days


Bd Infection Abundance (Log (1 + genomic equivalents))
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Bd Exposure at Cold Temperature
Bd Exposure at Warm Temperature
Constant
Shifted
Constant
Shifted
Red Legged Frog
Western Toad